Heterozygosity at a single locus explains a large proportion of variation in two fitness-related traits in great tits: a general or a local effect?

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Abstract: In natural populations, mating between relatives can have important fitness consequences due to the negative effects of reduced heterozygosity. Parental level of inbreeding or heterozygosity has been also found to influence the performance of offspring, via direct and indirect parental effects that are independent of the progeny own level of genetic diversity. In this study, we first analysed the effects of parental heterozygosity and relatedness (i.e. an estimate of offspring genetic diversity) on four traits related to offspring viability in great tits (Parus major) using 15 microsatellite markers. Second, we tested whether significant heterozygosity–fitness correlations (HFCs) were due to 'local' (i.e. linkage to genes influencing fitness) and/or 'general' (genome-wide heterozygosity) effects. We found a significant negative relationship between parental genetic relatedness and hatching success, and maternal heterozygosity was positively associated with offspring body size. The characteristics of the studied populations (recent admixture, polygynous matings) together with the fact that we found evidence for identity disequilibrium across our set of neutral markers suggest that HFCs may have resulted from genome-wide inbreeding depression. However, one locus (Ase18) had disproportionately large effects on the observed HFCs: heterozygosity at this locus had significant positive effects on hatching success and offspring size. It suggests that this marker may lie near to a functional locus under selection (i.e. a local effect) or, alternatively, heterozygosity at this locus might be correlated to heterozygosity across the genome due to the extensive ID found in our populations (i.e. a general effect). Collectively, our results lend support to both the general and local effect hypotheses and reinforce the view that HFCs lie on a continuum from inbreeding depression to those strictly due to linkage between marker loci and genes under selection.

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Heterozygosity at a single locus explains a large proportion of variation in two fitness-related traits in great tits: a general or a local effect?

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Abstract

In natural populations, mating between relatives can have important fitness consequences due to the negative effects of reduced heterozygosity. Parental level of inbreeding or heterozygosity has been also found to influence the performance of offspring, via direct and indirect parental effects that are independent of the progeny own level of genetic diversity. In this study, we first analysed the effects of parental heterozygosity and relatedness (i.e. an estimate of offspring genetic diversity) on four traits related to offspring viability in great tits (*Parus major*) using 15 microsatellite markers. Second, we tested whether significant heterozygosity–fitness correlations (HFCs) were due to 'local' (i.e. linkage to genes influencing fitness) and/or 'general' (genome-wide heterozygosity) effects. We found a significant negative relationship between parental genetic relatedness and hatching success, and maternal heterozygosity was positively associated with offspring body size. The characteristics of the studied populations (recent admixture, polygynous matings) together with the fact that we found evidence for identity disequilibrium across our set of neutral markers suggest that HFCs may have resulted from genome-wide inbreeding depression. However, one locus (*Ase18*) had disproportionately large effects on the observed HFCs: heterozygosity at this locus had significant positive effects on hatching success and offspring size. It suggests that this marker may lie near to a functional locus under selection (i.e. a local effect) or, alternatively, heterozygosity at this locus might be correlated to heterozygosity across the genome due to the extensive ID found in our populations (i.e. a general effect). Collectively, our results lend support to both the general and local effect hypotheses and reinforce the view that HFCs lie on a continuum from inbreeding depression to those strictly due to linkage between marker loci and genes under selection.

Introduction

Inbreeding is frequently evoked as one of the major threats to small natural populations due to the associated loss of individual genetic diversity and fitness (Reed & Frankham, 2003). In this context, understanding the relationship between genetic diversity and fitness-related traits constitutes a key aspect as it allows, among other things, to predict the consequences of a reduction in heterozygosity levels and evaluate the viability of populations (Ellegren & Sheldon, 2008). Progeny of related individuals may have reduced fitness as consequence of both the expression of deleterious or partly deleterious recessive alleles and the loss of

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heterozygosity advantage for genes experiencing balancing selection (Charlesworth & Charlesworth, 1987; Keller & Waller 2002). Accordingly, there is compelling evidence about the negative effects of inbreeding and reduced genetic diversity on the performance of individuals through impaired growth (Kruuk *et al.*, 2002; Bean *et al.*, 2004), lower resistance to disease (Reid *et al.*, 2007) or reduced neonatal or post-natal survival (Coltman *et al.*, 1998; Hansson *et al.*, 2001; Van de Casteele *et al.*, 2003; Mainguy *et al.*, 2009) for a variety of taxa. In addition, parent's genetic diversity can affect the fitness of their progeny irrespective of offspring genotype. Regarding the latter, highly heterozygous mothers can allocate more resources (hormones, antimicrobial proteins or nutrients) to their progeny during development and this result in increased offspring fitness (reviewed in Nager, 2006; Krist, 2011). However, evidence for an association between maternal heterozygosity and offspring fitness *via* maternal effects is scarce (see Brouwer *et al.*, 2007 for an exception). On the other hand, offspring viability could be also affected through the rearing environment created by parents, for example *via* parental care (Richardson *et al.*, 2004). Inbred individuals may exhibit reduced incubation expenditure (Pooley, 2013) or they may be less able to devote energy to a highly demanding activity as food provisioning (García-Navas *et al.*, 2009). Both maternal and paternal heterozygosity/inbreeding effects may explain why some studies have found lower survival probability in descendants from inbreed individuals independent of the effects of mate relatedness (i.e. offspring own level of inbreeding or genetic diversity) (Keller, 1998; Marr *et al.*, 2006).

The effects of inbreeding should be ideally assessed from inbreeding coefficients derived from well-resolved pedigrees (Pemberton, 2004). However, this information is very hard to obtain in wild populations and, as a result, it is only available for a few number of small and isolated populations (e.g. Keller, 1998; Richardson *et al.*, 2004) and much more limited in open populations (e.g. Szulkin *et al.*, 2007). The use of molecular markers is a widely used alternative to obtain indirect estimates of inbreeding, and there is a large body of literature reporting the existence of positive associations between heterozygosity and fitness-related traits (i.e. heterozygosity–fitness correlations, HFCs) (Coltman & Slate, 2003; Chapman *et al.*, 2009). As properly noted by Chapman *et al.* (2009), an important issue when designing a HFC study (or a study on inbreeding depression) is the choice of the variables to be used as fitness surrogates. Early life-history traits are considered to be polygenic and targets for deleterious recessive mutations, constituting good examples of characters suitable for this kind of study (Houle, 1998). In this sense, early-life stages are especially susceptible to the negative effects caused by reduced genetic diversity because it is expected that major genes are expressed early in development and early-acting traits associated with fitness (e.g. embryo mortality) are subjected to strong natural selection (e.g. Bensch *et al.*, 1994; Pujolar *et al.*, 2006; Rijks *et al.*, 2008; Mainguy *et al.*, 2009). In addition, selection at early-life stages is likely to reduce variance in inbreeding and hide the relationship between genetic diversity and fitness components later in life (Keller & Waller 2002, Hansson, 2004). This could be attributed to differential mortality removes the most inbreed/homozygous individuals from the population as consequence of the effects of lethal or sublethal alleles that are likely to be responsible for inbreeding depression in traits associated with fitness during development or early in life (Hemmings *et al.*, 2012).

HFCs have been explained by two main mechanisms. The first hypothesis is that HFCs occur because the set of employed markers reflect genome-wide levels of heterozygosity and they are able to capture the variance in levels of inbreeding present within the study population (David, 1998; Szulkin *et al.*, 2010). This happens because departures from random mating (e.g. inbreeding) or genetic drift (e.g. population bottlenecks or other demographic events) can generate correlations in heterozygosity and/or homozygosity across loci distributed genome-wide, a phenomenon termed identity disequilibrium (ID) (Slate *et al.*, 2004; Szulkin *et al.*, 2010). Although ID is considered to be the main cause of the existence of associations between heterozygosity and fitness traits, the conditions under which HFCs are expected to be associated with genome-wide inbreeding are thought to be rather restrictive (Balloux *et al.*, 2004). Studies based on simulated and empirical data have suggested that it would be necessary strong variance in inbreeding (e.g. favoured by high levels of polygyny or strong population structure), population admixture and/or bottlenecks to achieve a significant correlation between heterozygosity estimated at a few markers and genome-wide heterozygosity, situations most of which are generally likely to be uncommon in natural and open populations (Keller & Waller 2002, Slate *et al.*, 2004; Balloux *et al.*, 2004; Szulkin *et al.*, 2010).

Secondly, the 'local effect' hypothesis states that HFCs occur due to linkage disequilibrium (LD), a term used to refer to the nonrandom association of alleles at linked loci, between genotyped markers and nearby coding loci displaying overdominance or carrying deleterious recessive alleles (David, 1998). So, under this 'local effect' hypothesis, apparent heterozygote advantage results from genetic associations between the neutral markers and linked loci under selection (Hansson & Westerberg, 2002). Despite local effects are expected to be very hard to detect (Szulkin *et al.*, 2010), there is increasing evidence in support of this hypothesis and some studies have shown that one or a few neutral loci contribute more to HFCs than others (Hansson *et al.*, 2004; Brouwer *et al.*, 2007; Da Silva *et al.*, 2009). However, this is expected by chance even under the

"general effect" hypothesis, and therefore, the identification of significant local effects requires the application of appropriate statistical tests (Szulkin *et al.*, 2010; Olano-Marín *et al.*, 2011a). Thus, both models (general and local effects) are nonmutually exclusive and partly as consequence of this, the underlying mechanisms causing HFCs are not yet well understood and such apparent dichotomy is matter of ongoing controversy (Szulkin *et al.*, 2010; Olano-Marín *et al.*, 2011b).

In the present study, we examine the relationship between individual genetic diversity and several aspects of reproductive performance in two Mediterranean great tit (*Parus major*) populations monitored over five study years and genotyped at 15 polymorphic microsatellite markers. Specifically, we sought to test (i) whether there is an association between genetic diversity (parental heterozygosity, parental relatedness) and four fitness traits related with different components of offspring viability (hatching success, offspring size, offspring condition and number of fledged young) and (ii) whether the existence of HFCs is due to genome-wide or local effects.

Material and methods

Study system and field procedures

Between 2009 and 2013, we monitored two nearby populations of great tits breeding in nest boxes at Quintos de Mora (Montes de Toledo, central Spain). Each nest-box plot (Gil García: 39°22′N 4°07′W; Valdeyernos: 39°26′N 4°05′W) contains 100 wooden nest boxes erected across 20–25 ha of deciduous forest and Mediterranean scrubland. Both sites are separated by 7 km (see García-Navas *et al.*, 2014 for more details about the study area). During the breeding season, starting before nest-building (early April) and continuing until the chicks fledged (mid-June), we monitored the social pairing and the breeding success of these nest-box populations. Adults were captured using spring-traps, sexed, aged (as 1st year breeder or older) according to plumage characteristics and banded with metal rings. Blood samples from the parents were collected by puncturing the brachial vein and stored on FTA cards (Whatman Bioscience, Florham Park, NJ, USA). On day 13 post-hatching, nestlings were measured to the nearest 0.01 mm (tarsus length) and weighed to the nearest 0.1 g using a digital calliper and an electronic portable balance, respectively. All morphometric measurements were taken by the same person (VGN).

Laboratory methods

We genotyped great tits across 16 putatively neutral (*sensu* Olano-Marín *et al.*, 2011a) microsatellite loci (see Supporting Information). Genomic DNA was isolated using commercial kits (NucleoSpin Blood, Macherey-Nagel; GmbH & Co, Düren, Germany). Approximately 1 ng of template DNA was amplified in 10 μL reaction volumes containing 1× reaction buffer (67 mM Tris-HCl, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20; Eco-Start Reaction Buffer, Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, NED, PET or VIC) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The PCR profile consisted of 9 min of initial denaturing at 95 °C followed by 40 cycles of 30 s at 94 °C, 45 s at the annealing temperature (see Supporting Information) and 45 s at 72 °C, ending with a 10-min final elongation stage at 72 °C. Amplification products were run on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and fragment size was determined using GENEMAPPER 3.7 (Applied Biosystems).

Basic genetic statistics

All microsatellite loci were tested for deviation from Hardy–Weinberg (HW) and linkage disequilibrium (LD) using the software GENEPOP *on the web* (http://genepop.curtin.edu.au/; Rousset, 2008). Significance was assessed by applying a Markov chain method using 100 batches and 1000 iterations per batch. The degree of LD between all pairs of loci, estimated as the correlation coefficient (*r*LD) between alleles at different loci, was computed with the program LINKDOS *on the web* (http://genepop.curtin.edu.au/linkdos.html; Garnier-Gere & Dillmann, 1992). To test the significance of *r*LD, we used the exact genotypic disequilibrium test available in GENEPOP (Rousset, 2008). In order to account for multiple testing, we applied a Holm–Bonferroni correction (Rice, 1989) using the 'p.adjust' function (method = 'holm') in R (R Development Core Team, 2012).

Population genetic structure

It has been pointed out that sampling individuals from different localities or geographic origins can lead to spurious associations between heterozygosity and fitness-related traits (*sensu* Slate *et al.*, 2004; Slate & Pemberton, 2006). Thus, we examined whether population stratification may be a confounding factor in our HFC analyses. Specifically, we tested whether these populations are genetically differentiated or whether there is a high level of population admixture (i.e. no population substructure). The degree of genetic differentiation between populations was quantified using Weir and Cockerham's standardized F_{ST} (Weir & Cockerham, 1984). We calculated the pairwise F_{ST} value between the two populations and tested its significance with a Fisher's exact test after 9 999 permutations using GenAlEx version 6.5 (Peakall & Smouse, 2012). We also analysed patterns of genetic structure using a Bayesian Markov chain Monte Carlo clustering analysis implemented in the program STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). We ran STRUCTURE assuming

correlated allele frequencies and admixture and using prior population information (Hubisz *et al.*, 2009). We conducted ten independent runs for each value of $K = 1–5$ to estimate the "true" number of clusters with 200 000 MCMC iterations, following a burn-in step of 100000 iterations. The number of populations best fitting the data set was defined using the value of K at which Pr(X|K) (an estimate of the posterior probability of the data for a given K; Pritchard *et al.*, 2000) reached a plateau or continued to increase slightly. We used STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to compile and visualize the results from STRUCTURE runs. Lastly, we tested explicitly for differences in heterozygosity between both sites using a one-way ANOVA in STATISTICA 7 (Statsoft Inc., Tulsa, OK, USA).

Heterozygosity and parental relatedness

Heterozygosity was calculated for each genotyped individual ($n = 174$) using two different metrics: standardized multilocus heterozygosity (st*MLH*; Coltman *et al.*, 1999) and homozygosity by loci (*HL*; Aparicio *et al.*, 2006). st*MLH* is calculated as the number of loci that are heterozygous divided by the total number of typed loci. This measure avoids any potential bias that may be introduced by missing data at particular loci. *HL* improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value depending on its allelic variability. st*MLH* and *HL* were calculated using an Excel macro written by W. Amos (www.zoo.cam.ac.uk/departments/molecular-ecology/IRmacroN4.xls). These two heterozygosity estimates were highly correlated ($r = -0.95$, $p < 0.001$). Thus, for simplicity and in order to be consistent with previous studies (Ortego *et al.*, 2007, 2008, 2009; García-Navas *et al.*, 2009), we only present results for *HL*. Our results remained similar using st*MLH* (analyses not shown).

We used pairwise relatedness as an estimate of the coefficient of kinship between two individuals (i.e. the proportion of alleles shared between them). We calculated parental relatedness estimated as the Queller & Goodnight's (1989) coefficient (*rQG*) using the program COANCESTRY (Wang, 2011). Queller & Goodnight's r reflects the genotypic similarity of loci between a pair in comparison with the expected value between two individuals selected at random from the population. Hence, when the r coefficient is negative, it means that the relatedness between the pair was lower than that expected between two random individuals (Queller & Goodnight, 1989).

Identity disequilibrium and expected power to detect inbreeding

Correlation in heterozygosity and/or homozygosity across loci, which is commonly known as identity disequilibrium, is considered to be the fundamental cause of HFCs (Szulkin *et al.*, 2010). Different methods have been proposed to test the efficacy of given set of molecular markers in detecting genome-wide heterozygosity, and ultimately the individual inbreeding level (Slate *et al.*, 2004). We used two approaches to test the significance of identity disequilibrium. First, we calculated 'heterozygosity–heterozygosity correlations' (HHC), following Balloux *et al.* (2004). If our set of microsatellite markers carries information about genome-wide levels of heterozygosity, then comparing two random subsets of such markers should yield a positive significant correlation (Balloux *et al.*, 2004). The mean correlation between the two sets is interpreted as the HHC coefficient (rHHC). We ran 1000 randomizations of the markers to estimate the average rHHC and their respective 95% confidence intervals for each population using the R package 'Rhh' (Alho *et al.*, 2010). Complementarily, we also calculated the excess of double heterozygous at two loci relative to the expectation of random association standardized by average heterozygosity, which is expressed by means of the parameter g^2 (David *et al.*, 2007). This estimate is constant for any pair of loci considered and only depends on the mean and variance of inbreeding in the population (David *et al.*, 2007; Szulkin *et al.*, 2010). We used RMES (Robust Multilocus Estimate of Selfing; http://www.cefe.cnrs.fr/en/genetique-et-ecologie-evolutive/patrice-david) software to calculate g^2 and test whether this parameter differed significantly from zero.

When pedigree information is lacking or incomplete, marker-based estimates of genetic diversity (e.g. *HL* or st*MLH*) constitute an alternative to infer inbreeding coefficients (f) of individuals. However, previous studies have shown that the strength of the association between f and *MLH*, which depend on the demographic history and prevailing mating system of the population, is generally weak (Balloux *et al.*, 2004; Slate *et al.*, 2004). We used the equation (eqn 5) provided by Miller *et al.* (2014) to estimate the power of our markers to estimate inbreeding in our study system. According to the Miller *et al.*'s equation, the correlation between f and *MLH* is a function of the number of loci considered, their average heterozygosity and the magnitude of ID as measured by g^2 (Miller & Coltman, 2014).

Heterozygosity–fitness correlations: multilocus effects

We used mixed-effects models to analyse the association between genetic diversity (parental heterozygosity and pairwise relatedness) and four fitness-related traits: hatching success (calculated as the proportion of eggs laid that hatched), fledgling success (proportion of eggs that resulted in fledged young), offspring size (estimated as mean tarsus length) and offspring condition (mean body mass corrected for tarsus length). First, we

constructed a full model including the fitness-related trait as dependent variable and a series of genetic (see above) and nongenetic (e.g. study year and biologically relevant variables such as brood size or female/male age; see Table 1 for details) terms. Although we found no evidence for genetic subdivision across the whole study area (see Results), we followed the conservative criterion of fitting population identity as fixed factor into the models. Female, male and breeding pair identities were included as random effects to control for multiple breeding attempts. Most HFC studies have reported a linear relationship between fitness and genetic diversity, implying directional selection on heterozygosity. However, heterozygosity can also be under stabilizing selection with highest fitness corresponding to intermediate values of heterozygosity (e.g. Aparicio *et al.*, 2001). Therefore, we included both *HL* (or *rQG*) and its quadratic term in our analyses. Hatching and fledgling success were modelled as a binomial response variable where the binomial numerator (event) was the number of successes (number of hatched/fledged young) and the denominator (trial) was the number of successes in the previous stage (number of laid eggs/number of hatched eggs). For the analysis of nestling tarsus length, we additionally fitted the mean tarsus length of the two parents (mid-parent mean tarsus length) into the model to account for the heritability of this trait (Riddington & Gosler, 1995). It should be noted that for the other studied traits, the level of resemblance between parents and offspring is typically very low (h^2 = 0.1–0.2; Merilä & Sheldon, 2000). We were unable to identify extra-pair offspring as blood sampling, and microsatellite genotyping of nestlings is not routinely conducted in our study populations. However, paternity analyses conducted in a small subsample of nests (100 nestlings from 15 nests in 2012 breeding season) indicate that the incidence of extra-pair young in this population is moderately low (17%; V. García-Navas. *unpubl. data*). All nonsignificant variables were removed from the full models by adopting a backward-stepwise selection procedure. Analyses were conducted using SAS 9.1 (SAS Institute, Cary, NC, USA).

Table 1 Analyses of early-life fitness-related traits in relation to genetic and nongenetic factors. Only significant variables retained in the final model plus the variables of main interest (mother heterozygosity, father heterozygosity, parental relatedness) are shown. Each model initially also included all the variables indicated in the lists of 'rejected terms'. We tested for quadratic effects of heterozygosity and relatedness in all models, but these were never significant and are not presented. Female, male and breeding pair identities were fitted as random effects. Significant variables ($p < 0.05$) are denoted in bold.

Trait	Explanatory terms	Estimate ± SE	Test	*P*
Hatching success[a]	Intercept	−7.95 ± 0.26		
	Laying date	0.05 ± 0.01	$Z_{1,126} = 5.01$	**<0.001**
	Mother heterozygosity		$Z_{1,101} = -0.27$	0.78
	Father heterozygosity		$Z_{1,91} = 0.69$	0.49
	Parental relatedness	−2.19 ± 0.74	$Z_{1,63} = -2.97$	**<0.01**
	Rejected terms: year, population, mother/father age			
Fledgling success[b]	Intercept	−6.78 ± 0.34		
	Laying date	0.03 ± 0.01	$Z_{1,99} = 3.12$	**<0.001**
	Mother age	0.74 ± 0.25	$Z_{1,99} = 2.92$	**<0.01**
	Mother heterozygosity		$Z_{1,97} = 1.63$	0.10
	Father heterozygosity		$Z_{1,89} = 0.92$	0.35
	Parental relatedness		$Z_{1,66} = 1.18$	0.23
	Rejected terms: year, population, father age			
Offspring size[c]	Intercept	11.51 ± 1.75		
	Mid−parent tarsus length	0.41 ± 0.09	$F_{1,55.5} = 20.52$	**<0.001**
	Mother heterozygosity	−0.83 ± 0.36	$F_{1,39.8} = 5.15$	**0.028**
	Father heterozygosity		$F_{1,51.6} = 0.01$	0.98
	Parental relatedness		$F_{1,36.1} = 0.12$	0.73
	Rejected terms: year, population, laying date, brood size, mother/father age			
Offspring body mass[d]	Intercept	−9.84 ± 4.62		
	Offspring tarsus length	1.40 ± 0.24	$F_{1,70} = 33.98$	**<0.001**
	Mother heterozygosity		$F_{1,40} = 0.85$	0.36
	Father heterozygosity		$F_{1,41.6} = 0.83$	0.36
	Parental relatedness		$F_{1,53.8} = 0.01$	0.96
	Rejected terms: year, population, laying date, brood size, mother/father age			

[a]Number of hatched eggs (nominator)/clutch size (denominator).
[b]Number of fledged young (nominator)/number of hatched eggs (denominator).
[c]Mean tarsus length.
[d]Body mass corrected for skeletal size (tarsus length).

Heterozygosity–fitness correlations: single-locus effects

To test for the possibility that local, rather than general, effects were behind the observed HFCs, we built two different models for those cases where the final model included a measure of genetic diversity: (i) a multiple regression model with the multilocus estimator (*HL* or *rQG*) as the sole predictor and (ii) a multiple regression model including all single-locus heterozygosities (*SLH*) or single-locus relatednesses (*SLrQG*) terms fitted as explanatory variables. In the case of *SLH* models, each locus was included as an individual predictor (coded as 0 or 1 for homozygous or heterozygous, respectively). As there are large differences in variability among the employed loci (Table S1), we also performed this test considering standardized heterozygosities (i.e. giving more weight to more heterozygous loci) in the *SLH* model following to Szulkin *et al.* (2010). Similarly, we calculated relatedness values considering each locus separately; so, we obtained 14 different relatedness estimates for each social pair. In both cases, missing data were filled with a constant (the average heterozygosity or mean relatedness value for that locus obtained from all individuals successfully scored at that locus) following Szulkin *et al.* (2010). We tested whether the two models (i.e. multilocus *vs*. single locus) differed significantly from each other using an *F*-ratio test (Szulkin *et al.*, 2010). If the single-locus model explains more variance than the multilocus model, then this lends support to the "local effect" hypothesis (David, 1997; Szulkin *et al.*, 2010). Finally, we tested whether the absolute effect size of *SLH* was correlated with marker diversity (estimated as expected heterozygosity, H_E, observed heterozygosity, H_O, and allelic richness, A_R; see Table S1 in Supporting Information) and whether these loci show a similar effect size for the different studied traits.

Results

Basic genetic statistics

We genotyped 88 females and 86 males across a panel of 16 microsatellite loci. One locus (*Escu6*) deviated significantly from HW equilibrium in both populations and was excluded from further analyses. The number of alleles per locus ranged from 3 to 34, and the expected and observed heterozygosity ranged from 0.14 to 0.90 (see Table S1 in Supporting Information for more details). After correcting for multiple tests, we only found significant LD for the pair of loci *PmaTGAn33*/*Pca9* in Gil García (*r*LD = 0.06), *Pat-MP2-43*/*Mcyμ4* in Valdeyernos (*r*LD = 0.36) and *PmaTGAn33*/*Ase18* in both populations (*r*LD = 0.07 and 0.13 in Gil García and Valdeyernos, respectively) (all *q*-values <0.001). As we found no consistent LD across the two study populations or the LD correlation coefficient (rLD) between these pairs of loci was very small, none of these markers was discarded.

Spatial genetic structure

The obtained F_{ST} value indicate the absence of significant genetic differentiation between the two studied populations (F_{ST} = 0.006, P = 0.22). STRUCTURE analyses revealed a maximum Pr(X|K) value at K = 1 and thereafter decreased slightly (for K = 2) and then steeply (for K > 2), indicating support for a single genetic cluster (see Supporting Information for details). These results indicate that the two studied populations are not genetically differentiated. This lack of genetic structure is in agreement with capture–mark–recapture data, which have revealed both natal and breeding dispersal movements between the two populations (in both directions and by both sexes; V. García-Navas, *unpubl. data*). In addition, we did not find significant differences in individual heterozygosity between sites (Gil García: 0.29 ± 0.01, Valdeyernos: 0.28 ± 0.02; F = 0.27, P = 0.59). Thus, we can rule out the possibility of cryptic population stratification (*sensu* Slate & Pemberton, 2006) as an explanation for the observed HFCs in our study system (see below).

Identity disequilibrium and expected power to detect inbreeding

We found a positive and significant correlation between randomly assigned subsets of loci following the method of Balloux *et al.* (2004) (r = 0.41, 95% CI = 0.322–0.510). We also analysed HHC for each population separately; we obtained a positive correlation in both cases, but such relationship only was statistically significant (i.e. 95% credible intervals did not cross zero) in one of them (Gil García: r = 0.17, 95% CI = 0.037–0.287; Valdeyernos: r = 0.08, 95% CI = −0.126–0.332). Additionally, we also computed the g^2 estimator of identity disequilibrium. This parameter differed significantly from zero when data from both populations were pooled (g^2 = 0.019, P < 0.01) as well as when individuals from Gil García (g^2 = 0.019, P < 0.01) and Valdeyernos (g^2 = 0.020, P = 0.01) were analysed separately. Thus, our results indicate that neutral marker heterozygosity is representative of genome-wide heterozygosity in this study system. According to the formula given in Miller *et al.* (2014), the expected correlation (r^2) between heterozygosity and f in our study system (joining both populations) is 0.37 (Gil García: 0.35; Valdeyernos: 0.40). In a recent review, Miller & Coltman (2014) reported that the average expected correlation between marker heterozygosity and inbreeding was 0.13 (range: 0–0.82, n = 50). Thus, the predicted correlation between *HL* and f here shown is well above the

average value obtained in previous studies (see Fig. 2 in Miller & Coltman, 2014).

Heterozygosity–fitness correlations: multilocus effects

Hatching success was significantly associated with parental relatedness after controlling for laying date (Table 1); the level of kinship negatively affected the proportion of hatched eggs (Fig. 1). There was no association between hatching success and maternal or paternal heterozygosity (Table 1). Fledgling success was not associated with parental heterozygosity or relatedness, but it was positively associated with other nongenetic terms (laying date and mother age; Table 1). We found that offspring size was positively associated with maternal heterozygosity after controlling for mid-parent size (Table 1); more heterozygous females produced chicks with larger tarsi than less heterozygous ones (Fig. 2). Neither paternal heterozygosity nor parental relatedness was significantly associated with this trait (Table 1). Offspring condition (size-corrected mass) was not significantly associated with any of the genetic terms, and only offspring size was retained in the final model (Table 1). Quadratic terms (HL^2 and rQG^2) were not significant in any model ($P > 0.2$). Finally, the interaction between parental heterozygosity/relatedness and population was not significant in any analysis (all P values > 0.25), indicating that the strength of the relationship between maternal heterozygosity and offspring size and between parental relatedness and hatching success did not differ between populations.

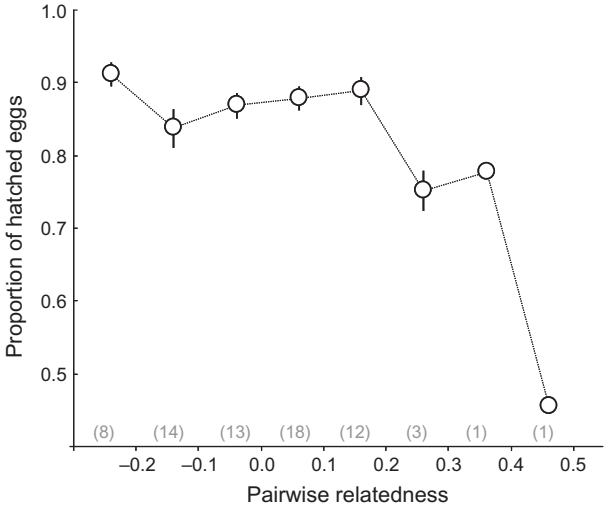

Fig. 1 Proportion of hatched eggs in relation to parental relatedness (n = 70 breeding pairs). Pairwise parental relatedness was categorized for illustrative purposes. Sample size for each category is given in parenthesis.

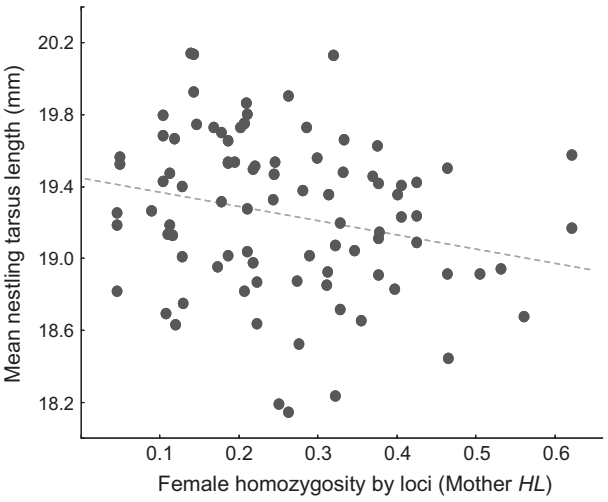

Fig. 2 Relationship between female homozygosity by loci (mother *HL*) and offspring size (mean nestling tarsus length). *HL* index ranges from 0 (when all loci are heterozygous) to 1 (when all loci are homozygous).

Heterozygosity–fitness correlations: single-locus effects

We estimated the importance of single-locus effects in the observed association between parental relatedness and hatching success. The *F*-ratio test revealed no significant difference between models, but the *SLH* model tended to explain a higher proportion of variance than the *MLH* model ($F_{14,52} = 1.64$, $P = 0.099$). We found an association between hatching success and single-locus pairwise relatedness estimated for one locus (*Ase18*; Fig. 3a), but the difference was nonsignificant after correcting for Bonferroni ($t = -2.26$, $P = 0.027$). More dissimilar pairs at locus *Ase18* had a higher hatching success than those that exhibited higher relatedness values at this locus (Fig. 3b). When we removed this locus from the calculation of parental relatedness values and ran another GLMM using this new variable, we found that the relationship between parental relatedness and hatching success still remained significant ($Z_{1,63} = -2.42$, $P = 0.015$).

We also examined whether the association between maternal multilocus heterozygosity and offspring size was caused better explained by single-locus effects. The *F*-ratio test showed that the *SLH* model did not improve the variance explained by the *MLH* model, but the difference was marginally significant ($F_{14,74} = 1.66$, $P = 0.083$). However, when employing standardized single-locus heterozygosities instead of raw heterozygosities, we obtained a significant result ($F_{14,74} = 1.91$, $P = 0.038$). Investigating the association between maternal heterozygosity at each locus and offspring size showed that, after correcting for multiple comparisons, locus *Ase18* was significantly associated with such

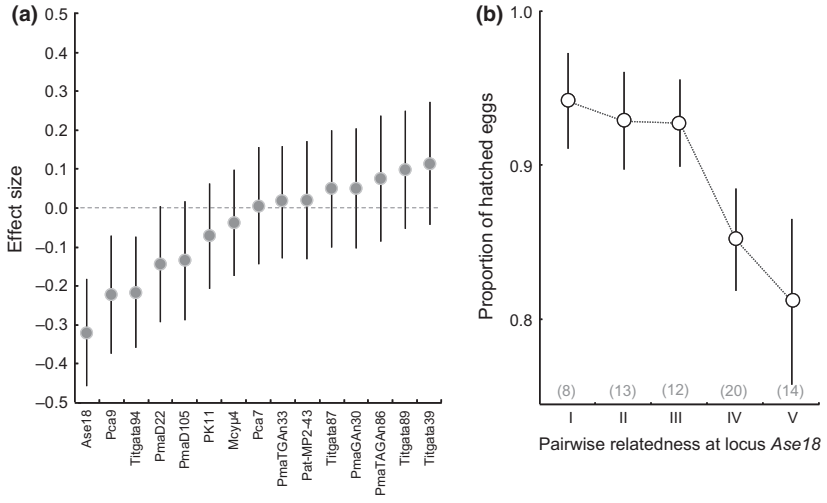

Fig. 3 (a) Effect sizes of single-locus parental relatedness for hatching success. (b) Relationship between hatching success and pairwise relatedness at locus *Ase18*. For illustrative purposes relatedness values were grouped in five different categories (*cat*. I: relatedness values from -1.5 to –1; *cat*. II: relatedness values from -1 to -0.5; *cat*. III: relatedness values equal to 0; *cat*. IV: relatedness values from 0.25 to 0.75; *cat*. V: relatedness values equal to 1). Sample size for each category is given in parenthesis.

variable ($t = 1.66$, $P = 0.0006$; Fig. 4a). Females heterozygous at locus *Ase18* produced larger chicks than those homozygous at this locus (Fig. 4b). When we removed this locus from the calculation of *MLH* and reanalysed our data using this new variable, we obtained a nonsignificant association between maternal heterozygosity and offspring size ($F_{1,48.1} = 2.31$, $P = 0.13$). It is worth to mention that the two loci (*Ase 18* and *PmaTGAn33*) that seem to have a greater influence on offspring size were in linkage disequilibrium but their effects went in opposite directions (Fig. 4a) (see above). When testing these loci separately, the effect of locus *Ase18* remained similar ($t = 3.73$, $P = 0.0003$), but the effect of locus *PmaTGAn33* disappeared ($t = -1.62$, $P = 0.11$).

Absolute effect size of *SLrQG* for hatching success was not correlated with marker genetic diversity (Spearman's correlation, H_E: $r_{15} = -0.31$, $P = 0.25$; H_O: $r_{15} = -0.33$, $P = 0.22$; A_R: $r_{15} = -0.30$, $P = 0.27$). Similarly, absolute effect size of *SLH* for offspring size was not correlated with none of these variables either (Spearman's correlation, H_E: $r_{15} = 0.14$, $P = 0.62$; H_O: $r_{15} = 0.13$, $P = 0.65$; A_R: $r_{15} = 0.15$, $P = 0.57$). The locus *Ase18* was not the most polymorphic one of our panel of loci; its variability (10 alleles) was below the average (14 alleles; see Supporting information). Absolute effect sizes of *SLrQG* for hatching success and *SLH* effect sizes for offspring size were not correlated ($r_{15} = -0.25$, $P = 0.35$).

Discussion

We found that hatching success decreased with mate relatedness and offspring size was positively associated with maternal heterozygosity. The association between offspring size and maternal heterozygosity was mainly explained by locus *Ase18*, suggesting the existence of a

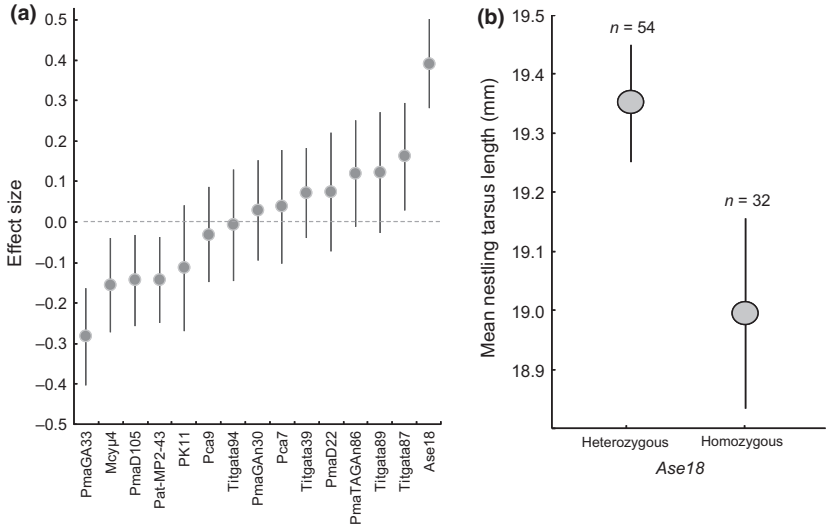

Fig. 4 (a) Effect sizes of single-locus heterozygosity (maternal genotype) for offspring size (mean nestling tarsus length). (b) Relationship between offspring size and maternal homozygosity and heterozygosity at locus *Ase18*.

local effect. In the case of the association between hatching success and parental relatedness, the same locus showed a disproportionate effect, but it did not exclusively explain the observed relationship. Hence, our results suggest that the association between genetic diversity and hatching success may be driven by a combination of both general and local effects, whereas variability at a single marker seems to be responsible for the observed correlation between maternal heterozygosity and offspring size (Szulkin *et al.*, 2010).

Identity disequilibrium and expected power to detect inbreeding

ID tests indicate that genetic diversity estimated at the 15 typed microsatellite markers may be representative of genome-wide heterozygosity and individual inbreeding coefficients. To our knowledge, this is one of the few studies reporting a significant g^2 value (Olano-Marín *et al.*, 2011a; Agudo *et al.*, 2012; Ruiz-López *et al.*, 2012; Annavi *et al.*, 2014). Analyses based on the method proposed by Balloux *et al.* (2004) confirmed this finding, as we found a significant heterozygosity–heterozygosity correlation between random sets of markers. Further, the predicted relationship between multilocus heterozygosity and f was above the average values obtained in previous studies with a similar or higher number of markers (see Table 3 in Grueber *et al.*, 2011). Thus, our study exemplifies that, in some circumstances, even a small number of microsatellites can be informative and provide enough power to reflect genome-wide heterozygosity and individual's inbreeding coefficients (Küpper *et al.*, 2010; Taylor *et al.*, 2010; Harrison *et al.*, 2011; Ruiz-López *et al.*, 2012; Forcada & Hoffman, 2014). For example, Jensen *et al.* (2007) found a similar correlation between heterozygosity and f to that reported by us using half of microsatellite markers (7 loci; $r = -0.38$) in an inbred population of house sparrow (*Passer domesticus*). In a recent study with blue tits (*Cyanistes caeruleus*), Olano-Marín *et al.* (2011a) found results similar to that obtained by Foerster *et al.* (2003) using an enlarged panel of loci (from 7 to 79) concluding that a relatively high number of microsatellites does not necessarily result in more power to detect HFC. In another recent study carried out with captive zebra finches (*Taeniopygia guttata*), Forstmeier *et al.* (2012) found that a panel of only 11 microsatellite markers produced about equally strong HFCs as a large panel of >1300 SNP markers (but see Hoffman *et al.*, 2014).

Our results contrasts with that of Chapman & Sheldon (2011) who failed to detect evidence for HFC in a noninbred great tit population using a set of 26 microsatellite markers. From the 15 microsatellites used in the present study, 6 (*Ase18* being one of them) were not included in the study of Chapman & Sheldon (2011). In this context, the particular conditions of each population (mating system, recent demography) have been identified as an important factor to be considered when designing and interpreting the results of HFCs studies (Szulkin *et al.*, 2010; Kardos *et al.*, 2014; Queiros *et al.*, 2014). In our study system, different circumstances may have contributed to increase ID and pose the necessary substrate upon which HFCs can arise. First, due to the shortage of natural cavities for nesting in the area, the studied populations can be considered as recently founded after the erection of nextboxes in 2006. This is likely to have enhanced genetic admixture if the original founders had different genetic backgrounds (i.e. if they belong to genetically differentiated populations), which may have contributed to increase population variance in genetic diversity and extensive ID (Szulkin *et al.*, 2010). Secondly, this species shows moderate levels of polygyny (Krokene *et al.*, 1998; Otter *et al.*, 2001; van Oers *et al.*, 2008; Szulkin *et al.*, 2012; V. García-Navas *unpubl. data*), which may have increased variance in inbreeding and the ability of neutral markers to predict individual's genome-wide heterozygosity (Balloux *et al.*, 2004).

Multilocus effects

Inbreeding often affects survival and other fitness-related traits more strongly during early-life stages than later in life (Keller & Waller 2002). For example, in birds, egg hatchability constitutes a trait especially vulnerable to inbreeding (Spottiswoode & Møller, 2004; Heber & Briskie, 2010). Our results support the body of evidence – from pedigree, genetic or experimental studies – suggesting that hatching success is often negatively affected by matings among relatives (e.g. Kempenaers *et al.*, 1996; Keller, 1998; Tregenza & Wedell, 2002; Van de Casteele *et al.*, 2003; Briskie & Mackintosh, 2004; but see Ortego *et al.*, 2010). We found a sudden decline in hatching success among the few pairs with high relatedness. Specifically, we observed a low hatching rate (78%) for those pairs that were related at or above the level of first cousins ($r > 0.1$) compared to that of pairs formed by nonkin ($r < 0$; 87%). A similar nonlinear relationship between hatching success and parental relatedness driven by a small proportion of the sample has been previously reported in great reed warblers (*Acrocephalus arundinaceus*) (Hansson, 2004) and collared flycatchers (*Ficedula albicollis*) (Kruuk *et al.*, 2002). Such nonlinear associations can arise if epistatic interactions between loci reinforce the negative effects of reduced genetic diversity among a small proportion of highly inbreed/homozygous individuals, a phenomena that would be likely to result in a threshold of genetic relatedness upon which the effects of reduced genetic diversity have lethal consequences on embryo development (see Fu & Ritland, 1996; Dudash *et al.*, 1997 and references therein). Alternatively, such pattern may arise if low-quality individuals are more likely

to mate with a relative and as a result they have lower hatching success. We can discard this hypothesis (non-random inbreeding with respect to phenotype) as we did not find a significant association between male or female size and genetic relatedness to the partner (r_{72} = −0.05, P = 0.67 and r_{72} = 0.11, P = 0.37, respectively).

We also found a significant relationship between mother heterozygosity and offspring size, a trait that is known to strongly affect post-fledgling survival in many passerines, including great tits (e.g. Garnett, 1981). This result may be explained by an increased parental care; for example, more heterozygous individuals may pose superior foraging skills or occupy better territories than homozygous individuals, which could positively affect offspring performance (Seddon *et al.*, 2004; García-Navas *et al.*, 2009). Alternatively, this effect may accrue through direct maternal effects if, for example, more heterozygous females supply more resources (e.g. hormones, antibodies) to their eggs. In this sense, several studies have reported associations between female heterozygosity and different aspects related to maternal egg allocation, including clutch size (Ortego *et al.*, 2007; García-Navas *et al.*, 2009; Olano-Marín *et al.*, 2011a), egg size (Wetzel *et al.*, 2012) and egg quality (shell spotting: García-Navas *et al.*, 2009; yolk mass: Pooley, 2013). In turn, egg size and egg quality have been also found to influence hatching probability and be important factors predicting offspring size in passerines (Sanz & García-Navas, 2009; Krist, 2011). Thus, it is possible that descendants of heterozygous females exhibit a superior phenotype (i.e. structurally larger offspring) due to increased maternal investment in eggs.

Single-locus effects

Comparing *MLH* and *SLH* models has been proposed as the best way to test for local effects (*F*-ratio test: David, 1997; Szulkin *et al.*, 2010). However, none of the studies that are often cited as showing evidence for the existence of a local effect applied the *F*-ratio test (Lieutenant-Gosselin & Bernatchez, 2006; Brouwer *et al.*, 2007; Da Silva *et al.*, 2009). In fact, to the best of our knowledge, no HFC study has passed such test and ours is the first one yielding significant results and providing partial support for the local effects hypothesis. Exploring the contribution of each locus, we found that the maternal heterozygosity–offspring size correlation was mainly due to locus *Ase18*. When we re-ran the *SLH* model including all loci except locus *Ase18*, this model produced a worse fit than the *MLH* model (*SLH*: adjusted-R^2 = 0.01, F = 1.09, P = 0.37 *vs.* *MLH*: adjusted-R^2 = 0.03, F = 4.28, P = 0.04). Furthermore, when removing locus *Ase18* from the calculation of *MLH*, the relationship between maternal heterozygosity and offspring size became nonsignificant, which implies that this particular locus had a disproportionate effect on our results. Hatching success showed a significant association with parental relatedness, and we also tested such trait for possible single-locus effects. The model including single-locus relatedness (*SLrQG*) as independent predictors was no better supported than the model including the multilocus estimator (*rQG*). Although, intriguingly, we observed that mate relatedness at locus *Ase18* had a strong and negative influence on hatching success. It allows us to suggest that this marker may lie near to a functional locus under selection and influencing these traits. The locus *Ase18* has been assigned to chromosome 3 of the zebra finch based on sequence homology (Warren *et al.*, 2010) and according to this predicted microsatellite map of the passerine genome, such locus is located near (5.6 kb distance) the gene SERTAD4. It is possible that, in the great tit, heterozygosity at this gene provides an advantage in one or more processes affecting the studied traits. However, we cannot but speculate about it since, regrettably, this gene's *in vivo* function is yet unknown. Alternatively, because of the existence of extensive ID in this population, heterozygosity at this locus (*Ase18*) might not only be correlated to heterozygosity in its own chromosomal region, but to heterozygosity across the genome. That is, the combined effects of many unlinked loci may override that of a few loci located in the chromosomal vicinity of the marker (Szulkin *et al.*, 2010).

Conclusions

Taken together, these results suggest that our set of 15 markers was powerful enough to reflect genome-wide heterozygosity and inbreeding in our study population. Our study highlights that under certain scenarios, a relatively modest number of marker loci (median number of markers in HFC studies is ~10, see Chapman *et al.*, 2009) can be useful to provide information about levels of inbreeding. We also found that one locus seems to have a disproportionate influence on the observed HFCs, which was particularly remarkable in the case of nestling size. Regarding this, a large part of studies in which the local effect hypothesis is claimed as the mechanism responsible for HFC came to this conclusion after failing to explain HFC by inbreeding. However, the general and local effect hypotheses are not mutually exclusive and they reflect the same phenomenon: the existence of deleterious recessives alleles and loci displaying overdominance dispersed throughout the genome (Szulkin *et al.*, 2010). Our results, thus, support the notion that, in practice, both mechanisms represent opposite ends of a broad spectrum that runs from 'classical' inbreeding through to chance linkage between a marker and few genes of large effects (Balloux *et al.*, 2004). Finally, the advent of next-generation sequencing techniques and further studies simultaneously employing subsets of putatively neutral

and functional markers (Szulkin & David, 2011; e.g. Olano-Marín *et al.*, 2011a,b) can solve imminently some of the above mentioned problems (e.g. the employment of a huge number of loci and identification of key genes) and open new avenues of research into the underlying mechanisms of HFCs (Szulkin & David, 2011; Hoffman *et al.*, 2014).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Panel of 16 polymorphic microsatellite markers used in this study.

Appendix S1 Supplementary references.

Figure S1 Results of the STRUCTURE analyses showing the mean (± SE) Ln Pr(X|*K*) based on ten runs (replicates) for each value of *K*.

Figure S2 Results of the genetic assignment based on STRUCTURE analyses for $K = 2$.

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